IMMUNOLOGIC STUDIES ON DUCK-EMBRYO-PROPAGATED NEWCASTLE DISEASE VIRUS

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Interest in Newcastle disease was stimulated in the United States by the comprehensive review of the literature on this poultry infection and attempts at immunization published by Beaudette (1943).

The Maktesar strain of Ranikhet disease virus was attenuated by chick-embryo-passage and was the first live virus Newcastle disease vaccine to be used extensively. Haddow (1946) reported a mortality rate of from 2.5 to 6 per cent in the first 100,000 birds to receive this vaccine in India.

Brandly, Moses, Jones and Jungherr (1946) reported experiments with several types of vaccines. The addition of formalin or irradiation by ultra-violet light provided highly immunogenic inactivated vaccines. Evaluation of immunity response was accomplished by serum-virus neutralization tests in embryonating eggs and challenge inoculation with large doses of virus.

Brandly, Moses and Jungherr (1946) demonstrated the presence in egg yolk of specific antiviral antibodies in eggs from hens recovered from Newcastle disease or vaccinated with live vaccines. An antibody titer was found in chicks hatched from eggs of immune hens. This congenital passive immunity interfered materially with active immunity response to live virus for the first month of life and protected the chicks against large doses of virus for the first two weeks of life.

Komarov and Goldsmit (1946) serially passaged Newcastle disease virus in pigeons and ducklings. Fowls inoculated intradermally in the wattle with virus modified by ten intracerebral duck passages did not develop Newcastle disease and resisted virulent fowl virus challenge 18 days after vaccination.

Acevedo and Mendoza (1947) passed Newcastle disease virus up to 50 times in chicken, duck and turkey embryos without changing the virulence for chickens. They noted that the addition of 0.5 per cent of potassium alum to vaccines treated with 0.7 per cent sodium borate and 0.1 per cent formalin enhanced their antibody-producing properties. In a few trials the birds receiving 1 cc. doses of this vaccine in the legs were able to withstand inoculations of 500 to 1,000 m.i.d.'s of virus, while those given the vaccine without potassium alum were unable to do so.

Coronel (1947) reported the preparation of a highly satisfactory vaccine using alumina cream as an adsorbent and emulsifier and formalin as an attenuating agent. The minimum protective dose was 0.25 cc.

Vaccine with a pH of 6.8 to 7.2 had better keeping qualities than that with higher or lower pH values.

Millen (1947) gave an unpublished preliminary report on attenuation of California strain 11914 Newcastle disease virus in embryonated duck eggs. The first indication of attenuation came at the 20th passage when one of three chickens inoculated survived. The duck-embryo-passaged virus produced HI antibodies and was protective against virulent virus challenge. An experimental adsorbate vaccine produced HI titers in some vaccinated chicks and protected others with negative HI serum against virulent virus challenge. These HI negative chicks became HI positive upon challenge.

Komarov and Goldsmit (1947) reported that their Haifa strain (a duck-embryo-propagated virus) caused less paralysis and loss in egg production than the Maktesar strain when used to vaccinate laying flocks. The Haifa strain did not induce moulting.

Asplin (1947) found ducks and geese much more resistant to Newcastle disease than fowls. By inoculation high HI titers were produced in some ducks; and a high virus-neutralizing titer was also present in the same serum. Antibody production was detectable in both ducks and geese on the sixth and seventh days following inoculation. The appearance of antibody heralded the loss of infectivity.

Moses, Brandly and Jones (1947) evaluated the pH stability for four strains of Newcastle disease virus. The original virus material had a pH of 7.8 to 8.

Beach, Bankowski and Quortrup (1948) found that the virulence of three strains of Newcastle disease virus was not reduced during propagation for 28 generations in duck embryos by allantoic cavity inoculation or for ten generations by yolk sac inoculations. The virulence of one strain was appreciably enhanced for chickens by duck-embryo passage.

Schmittle and Millen (1948) modified the test for the hemagglutination-inhibition antibodies of Newcastle disease so that it could be applied to unincubated eggs.

Clancy, Cox and Bottorff (1949), using Van Roekel's MK107 strain of Newcastle disease virus, passaged it in eleven duck-embryo serial passages. Lyophilized virus material was restored and used to vaccinate laboratory fowls. The geometric mean HI titer was 1:2344 by the 13th day for 199 experimental chickens.

Makham, Cox and Bottorff (1949) described the preparation of vaccine from the 12th duck-embryo-passage of the MK107 strain. In field trials on 37,040 chicks the three-weeks post-vaccination loss was 1.363 per cent.

Materials and Methods

1. Experimental fowls

Experimental fowls, day-old chicks and hatching eggs were secured from Newcastle disease-free flocks. Fowls of all ages were kept in batteries and those receiving live virus or vaccines were isolated from unused stock and controls. Water and a complete poultry ration were provided *ad libitum*.

2. Newcastle disease virus strains

Four strains of Newcastle disease virus were passaged in embryonated duck eggs and used as live virus vaccines.

a. Strain D was California strain 11914 adapted to duck embryos in February, 1947.
b. Strain I was isolated from a mild outbreak of Newcastle disease in Illinois in June, 1948.
c. Strain L was taken from commercial vaccine supplied by Lederle Laboratories.
d. Strain D was supplied by Dr. F. R. Beaudette in August, 1948.

Challenge virus used was chick-embryo-propagated California 11914 virus originally secured from Dr. C. A. Brandly in July, 1946, and was designated as strain C.

3. Virus passage and storage

Ten-day embryonated duck eggs were inoculated in the allantoic cavity with diluted virus-containing allantoamniotic (a.a.) fluid with 0.1 cc. of inoculum used. For the first 33 serial passages of D virus White Pekin duck eggs were used. For the remainder of the passages White Indian Runner duck eggs were used. Virus from each egg was harvested separately and possible bacterial contamination was determined by inoculation of sterile blood agar media. Bacteriologically sterile a.a. fluid was stored in the frozen state in test tubes plugged with cotton. A six-cubic-foot freezer at −18° C. was used for virus storage after the 46th passage of strain D and for the other virus strains.

4. Virus titration

Titrations were made in ten-day chick embryos at every fifth passage. One-tenth of a cc. of material was used to inoculate each of three embryos for each ten-fold serial dilution of the virus in tryptone broth. The titer is the highest dilution in which at least two of the embryos were killed by the virus within six days' post-inoculation incubation. One-tenth of a cc. of this dilution is the embryo-lethal-dose (E.L.D.), or embryo minimum lethal dose, and is comparable to the LD50 dose of Reed and Muench (1938), being fractionally higher or lower depending

on whether one embryo survived in a lower dilution or one died in the next higher dilution. Each cc. of this dilution contains 10 E.L.D. A virus sample having a titer of 10^{-8} would therefore contain 10^{6} E.L.D. (1,000,000 embryo-lethal-doses) per cc. in a 10^{-3} dilution of embryonic fluids in tryptone broth (1 cc. a.a. fluid in 1000 cc. of diluent).

5. Adsorbed virus vaccine

The adsorbed virus used in this study contained a 10^{-3} dilution of a.a. fluid. The aluminum hydroxide was prepared in the desired concentration from borax and potassium alum and sterilized by boiling. An equal volume of 50 per cent glycerinated buffer made according to that used by Acevedo and Mendoza (1947) and a.a. fluid were used in the adsorbed virus. This amount of buffer had little effect on the pH of the vaccine compared to the effect of other ingredients but was added to determine whether it would interfere with adsorption, should a virus preserved in glycerinated buffer be used in place of a.a. fluid. The buffer possibly helped the initial solution of the dry ingredients but could be replaced by 1 gm. of dextrose for 1 cc. of buffer with no noticeable effect and could probably have been left out.

A 500 cc. sample was simply prepared as follows:

Distilled water	500 cc.
Borax	1.25 gm.
Potassium alum	1.5 gm.
Buffer	.5 cc

The mixture was heated to boiling and then placed in a sterile bottle plugged with cotton.

The resulting aluminum hydroxide suspension was approximately neutral in reaction. After the suspension was cooled in the refrigerator, 0.5 cc. of virus-containing a.a. fluid was shaken into the mixture. Samples to be formalinized were again shaken after the addition of 0.5 cc. of 40 per cent formalin.

6. Killed virus vaccine

Samples of allantoamniotic fluids from duck embryos killed with the virus were pooled for vaccine production. The final product contained ten per cent a.a. fluid, 5 per cent glycerinated buffer and 0.4 per cent formalin. One batch of whole embryo killed virus vaccine was made. The embryos were blended with part of the diluent in a Waring blender. The whole embryo concentration was diluted to 20 per cent.

7. Antiserum

Fowls that had been challenged with strain C virus were bled from the heart and the blood was immediately mixed with an anticoagulant

solution. This solution consisted of 200 cc. of distilled water, 7 gm. dextrose, 7 gm. sodium citrate, and 2 cc. of 40 per cent formalin for each liter of whole blood mixture. Any clots present were strained out with sterile gauze and the serum was separated from the cells in a small cream separator.

8. Vaccination technique

All vaccines, virus and antiserum described here were injected into the second wing section between the ulna and radius except in the cases of very young chicks and ducklings. These were injected subcutaneously in front of the thigh. Commercial products were injected into the site selected by the manufacturer.

The wing-web "stick" method of vaccination was used for some live virus vaccines.

Fowls becoming paralyzed so they could not stand were removed from the batteries and counted as having been killed by the virus.

9. Challenge

Usually fowls were challenged after the 15th post-vaccination day. Challenge virus consisted of a 10^{-3} dilution of strain C in buffered, distilled water or tryptone broth. A dose of 0.5 cc. for small chicks and one of 1 cc. for larger ones was injected in the second wing section.

Discussion

Evidence has been presented indicating that the duck embryo may be used to attenuate a strain of Newcastle disease virus, contrary to the findings of Acevedo and Mendoza (1947), who failed with 50 passages, and Beach, Bankowski and Quortrup (1948), who failed with three strains of virus during 28 generations. One of the three strains was the C·11914 which we also studied.

Komarov and Goldsmit (1947), Clancy, Cox and Bottorff (1949) and Makham, Cox and Bottorff (1949) found the duck embryo an effective means of propagating mild strains of Newcastle disease virus for live vaccine production but did not indicate any attenuation of the virus by duck-embryo passage.

Newcastle disease virus strain D, adapted from California strain 11914, was passaged in duck embryos for 101 serial generations. From the 20th passage some fowls inoculated with this virus survived and were resistant to subsequent challenge with the potent chick-embryo-propagated California 11914 strain. Even after 100 duck-embryo serial passages strain D caused fatal paralysis in part of the chicks receiving it as a live vaccine.

Virus isolated from a mild natural outbreak of Newcastle disease in Illinois was attenuated by duck-embryo passage. By the 20th generation this I strain compared favorably with duck-embryo-propagated commercial strains L and D.

The length of time required to kill inoculated duck embryos was apparently not an indication of the virulence of the virus for chickens. Strain D was developed as a 48-hour, 10-day, duck-embryo-killing virus, yet the milder strain B also killed 10-day duck embryos the second day. Day-old chicks, however, died in a shorter time when receiving California 11914 or strain D virus than when receiving the milder 1, B, or I strains.

Adsorbing Newcastle disease virus on dilute aluminum hydroxide and killing the unadsorbed virus with 0.1 per cent formalin reduced the virulence of the vaccine without preventing active immunization of a large proportion of the vaccinated chicks. The age of the chicks, virulence of the virus, pH of the medium and length of time of formalin treatment are factors in determining results. A relatively mild virus strain in a neutral medium, matured for 36 to 48 hours in a refrigerator, gave better immunizing results for very young chicks or laying hens than were obtained with mild live virus vaccination. This adsorbate vaccine resembles that made by Acevedo and Mendoza (1947) except that it contains less virus and aluminum hydroxide. The pH compares with that of 6.8 to 7.2 preferred by Coronel (1947). The adsorbent is made in a few minutes in the dilution desired. Both time and effort are saved over the two days required by Haddow and Idnani (1941) to produce their adsorbent, which was an improvement on previous methods requiring two weeks.

In limited trials with two brands of commercial killed vaccines and with experimental duck-embryo-propagated killed vaccine, the immunity produced was of a temporary nature. This finding is in accord with investigations reported by Beach (1944) and by Brandly, Moses, Jones, and Jungherr (1946). None of the killed vaccines tried produced HI antibodies.

Formalin-killed duck-embryo-propagated Newcastle disease virus, followed seven to 17 days later with live duck-embryo-propagated virus, induced the development of HI antibodies in vaccinated chicks and rendered them immune to subsequent challenge. Staub (1930) used a similar method to immunize chickens against fowl plague, except that the virus used by him was taken from infected spleen pulp. He recommended a 10-day period between the killed and live virus inoculations.

This dobule treatment is more expensive than the single adsorbate vaccination and cannot be used effectively on very young chicks or on

laying hens. Young chicks fail to develop sufficient immunity from the killed vaccine fully to protect them against the live virus and production may drop to nil following live virus inoculation in hens previously treated with killed vaccine. Newcastle disease antiserum with an HI titer above 1:1280 neutralized large doses of live virus injected simultaneously into chickens. However, in accord with results reported by Haddow (1936), Shirlaw (1937) and others who tried this method of immunization, many fowls so treated were susceptible when challenged later. The majority of fowls tested remained HI negative following simultaneous virus and antiserum injections, even when these injections were made separately on opposite sides of the chickens.

Delayed cessation of egg production in laying fowls receiving formalinized adsorbate vaccine and the ability to immunize chicks under two weeks of age by the same vaccine with subsequent HI antibody production suggested that a combination of antiserum and adsorbed live virus might overcome the weaknesses encountered in previous methods for vaccinating young chicks and hens in production. Trials on young chickens showed that 85 to 90 per cent of day-old chicks could be immunized by this method and that HI titers were produced in the blood of a majority of those tested. Chicks two weeks old and older could be immunized almost 100 per cent. This was also the safest method so far tried on laying fowls. Production in the 31-day post-vaccination period was maintained at 49.5 per cent in one group of 212 New Hampshire Reds and 46.3 per cent in a group of 13 White Rock pullets.

The antiserum apparently neutralizes the free virus but is used up before all the adsorbed virus is eluted. The antiserum and adsorbed live virus can apparently be mixed together and given as a single injection or administered separately with similar results. Vaccinated fowls gradually develop antibodies against the small amount of live virus as it is eluted and become actively and probably permanently immune. The appearance of the HI antibody in the blood or eggs is indicative that the fowl has reacted to live Newcastle disease virus.

Prior to the report of Schmittle and Millen (1948) studies on the antibody content of eggs from immune hens referred to the virus neutralization antibody. During this investigation it became desirable to follow the development of the HI antibody in vaccinated laying hens without submitting them to periodic bleeding; so the egg test was developed. Hens receiving live virus while in production laid HI negative eggs up until the time production was interrupted. The first egg laid when production was resumed was HI positive for each of 24 hens tested. Several pullets vaccinated before maturity laid several HI negative eggs before

HI positive eggs were produced, although their blood had been HI positive for several weeks.

Virus was isolated from eggs laid by two of ten vaccinated hens and from one of five eggs laid as a flock was going out of production in a natural outbreak of Newcastle disease. In both cases the eggs were incubated for ten days prior to chick-embryo inoculation of yolk material and did not contain live embryos when candled. In the case of the vaccinated hens the virus had been given four and five days previously and the last egg laid prior to the virus-containing eggs had live ten-day embryos in them when examined. Virus isolation from live embryos after ten days' incubation has not been reported. Removal of dead germ and infertile eggs after ten to 12 days' incubation followed by formaldehyde fumigation should prevent incubators from becoming contaminated with Newcastle disease virus.

Our results confirmed the findings of Brandly, Moses and Jungherr (1946) that the chicks hatched from hens recovered from the disease or vaccinated with live virus are refractory to live virus inoculation for at least two weeks but are fully susceptible at four weeks of age. Results from one trial on day-old chicks from HI positive hens using adsorbed live virus of three strains indicated that the adsorbed live virus was practically all neutralized by the passive immune antibodies of the chicks as fast as it was eluted until it had all been used up. Most of the chicks so treated died of Newcastle disease when challenged 43 days later.

The average pH of 7.7 found in this investigation for five strains of virus in the a.a. fluid of both chick and duck embryos is comparable to the pH of 7.8 to 8 reported by Moses, Brandly and Jones (1947) for the original chick-embryo virus material used by them in pH stability evaluations.

Natural occurrence of Newcastle disease in ducks was reported by Rodier (1928) and Farinas (1930) of the Philippines, Albiston and Gorrie (1942) of Australia and Andrews (1948) of England. Doyle (1927), Picard (1928), Ware (1932), Komarov and Goldsmit (1946) and Asplin (1947) used parenteral methods to secure infection. Asplin alone studied HI titers in inoculated ducks. In some of the ducks used by him high HI titers were present the sixth and seventh days following inoculation.

Crawford (1930), Kee (1928), Purchase (1931), Dobson (1939), Iyer (1945) and Millen and Bhatia (1946) found that ducks were resistant to natural exposure from infected fowls. Iyer also failed to produce the disease by parenteral inoculation of virus in Pennine ducklings.

In the investigation reported here HI titers were produced in the

blood of all six inoculated Muscovy ducks, *Carina moschata*. The HI antibody also was present in eggs laid by HI positive Muscovy ducks. The original virus strain C·11914 injected subcutaneously did not produce disease in White Pekin or White Indian Runner ducklings, *Anas boschas*. After duck-embryo modification of the virus fatal Newcastle disease was produced by subcutaneous inoculation in ducklings under a week of age. Part of the ducks over 30 days old receiving live Newcastle disease virus became HI positive. One strain of duck-embryo-modified virus was blindly passaged for five generations in ducklings at six-day intervals in a manner similar to that reported by Komarov and Goldsmit (1946) except that in their case the intracerebral route was used; in this investigation the virus was injected subcutaneously.

The duck is free of natural infection with fowl leukosis, pox and laryngotracheitis. The use of duck embryos, therefore, to propagate Newcastle disease and pox viruses for vaccine production may aid in preventing contamination of immunizing products with damaging strains of viruses which might be present in hen eggs.

Summary

1. The California strain 11914 of Newcastle disease virus was attenuated by 10-day duck-embryo passage. This modified "D" strain killed the majority of inoculated embryos on the second day.
2. Virus from a mild outbreak of Newcastle disease in Illinois was adapted to duck-embryo propagation. After 20 serial passages in duck embryos it was comparable to the two commercial strains "L" and "B" in safety and antigenic properties for production of live virus vaccines.
3. A method of preparing formalin-treated adsorbed virus vaccine relatively safe for young chicks and laying hens is described.
4. Live adsorbed virus with antiserum immunized 85 to 90 per cent of day-old chicks vaccinated and depressed production less than other vaccines tried on laying fowls.
5. The development of the HI antibody in a fowl indicates previous contact with live virus. The use of this test in both blood and eggs as a means of studying the efficacy of vaccines is indicated.
6. Chickens injected with formalin-killed, duck-embryo-propagated Newcastle disease virus followed seven to 17 days later by live duck-embryo-propagated virus became HI positive and resisted challenge with virulent chick-embryo-propagated California 11914 virus.
7. Newcastle disease virus strain C·11914 was modified by duck-embryo passage so that it produced symptoms and death in inoculated ducklings.
8. Virus was isolated from cases of experimental Newcastle disease in

ducklings and after further serial passage in bacteriologically sterile duck embryos was used to again produce fatal experimental Newcastle disease in ducklings.

9. Positive hemagglutination-inhibition antibodies for Newcastle disease virus were produced in six Muscovy ducks, *Carina moschata,* injected with live virus and HI antibodies were also found in eggs of HI positive Muscovy ducks.

10. HI antibodies were produced in some of the White Pekin and White Indian Runner ducks, *Anas boschas,* inoculated after they were 30 days old with live Newcastle disease virus.

11. Blind duck-to-duck serial passages of duck-embryo-propagated Newcastle disease virus were carried out at 6-day intervals for 5 passages without inducing observable symptoms in the subcutaneously inoculated ducks.

12. The pH of duck-embryo allanto-amniotic fluid containing Newcastle disease virus of five strains averaged 7.7 for 104 embryos. The pH of chick-embryo allanto-amniotic fluid containing virus of the same five strains also averaged 7.7 for 38 embryos.

VITA

The author was born February 5, 1908, at Monmouth, Illinois. He completed his elementary and high school education at Monmouth and received the B.S. degree from Monmouth College in 1930, with major work in chemistry.

After teaching for five years in Woodstock School for Missionaries' Children at Mussoorie, United Provinces, India, he began his veterinary course at Iowa State College, Ames. He was awarded the M.S. degree in entomology and the degree, Doctor of Veterinary Medicine, in 1939. During the period of his study at Iowa State College he was research assistant to the biological chemist at the Veterinary Research Institute and cooperative agent with the Bureau of Entomology, United States Department of Agriculture.

Before returning to India he spent one year in virus research with Allied Laboratories, Inc., Sioux City Division. From 1940 till 1946 he was under appointment by the Presbyterian Board of Foreign Missions and was stationed at the Allahabad Agricultural Institute, Allahabad, India, where he became Professor of Animal Husbandry and Dairying of Allahabad University. He was a member of the Editorial, Apiculture, Animal Husbandry, and Cattle Show Committees of the Imperial Council of Agricultural Research and was Associate Editor of the "Indian Bee Journal."

He joined the staff of the College of Veterinary Medicine of the University of Illinois in September, 1946, and at present holds the rank of Assistant Professor in the Department of Veterinary Pathology and Hygiene.

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